AD_________________

Award Number: W81XWH-11-1-0051

TITLE: Glutamate Signaling and Mitochnodrial Dysfunction in Models of Parkinson's Disease

PRINCIPAL INVESTIGATOR: D. James Surmeier, Ph.D.

CONTRACTING ORGANIZATION: Northwestern University
Evanston, IL 60208

REPORT DATE: December 2012

TYPE OF REPORT: Final, Option Year 1

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE
December 2012

2. REPORT TYPE
Final, Option Year 1

3. DATES COVERED
1 December 2011 – 30 November 2012

4. TITLE AND SUBTITLE
Glutamate Signaling and Mitochnodrial Dysfunction in Models of Parkinson's Disease

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0051

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
D. James Surmeier, Ph.D.

E-Mail: j-surmeier@northwestern.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Northwestern University
Evanston, IL 60208

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Our overall goal is to characterize the calcium-dependent interaction between intrinsically generated autonomous spiking and extrinsically generated glutamatergic synaptic transmission in shaping mitochondrial oxidant stress in substantia nigra dopaminergic neurons that are vulnerable in Parkinson's disease. In the last year, we have made excellent progress toward this goal. First, we found that SN dopaminergic neurons have a very low intrinsic buffering capacity for calcium, allowing it to readily diffuse between compartments. Second, sustained antagonism of voltage-dependent L-type calcium channels by systemic administration of drug, significantly lowers mitochondrial oxidant stress. Third, antagonizing glutamatergic NMDA receptors, but not metabotropic glutamate receptors, diminishes oxidant stress in dopaminergic neurons; stimulating NMDA receptors raises stress levels. Fourth, blocking receptors controlling the release of calcium from intracellular stores (IP3 and ryanodine receptors), diminished mitochondrial oxidant stress in pacemaking dopaminergic neurons, suggesting that calcium-induced-calcium-release (CICR) and injection of calcium into mitochondria was a key step in the elevation of oxidant stress; this inference is also consistent with our ability to attenuate stress by blocking the mitochondrial uniporter. We are now developing optical probes that will allow us to directly test this hypothesis. Lastly, we have developed a novel brain slice preparation that allows us to electrically activate pedunculopontine glutamatergic fibers synapsing upon dopaminergic neurons. Unexpectedly, these synapses have a high release probability, contrasting them with glutamatergic subthalamic nucleus synapses.

15. SUBJECT TERMS
glutamate receptor, calcium, mitochondria, oxidant stress, dopamine, substantia nigra

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	8	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Page

Introduction……………………………………………………………….……..….. **4**

Body…………………………………………………………………………….. **5**

Key Research Accomplishments…………………………………………….…….. **5**

Reportable Outcomes…………………………………………………………… **8**

Conclusion……………………………………………………………………… **8**

Appendices……………………………………………………………………… **n/a**

Introduction:
Our overall goal is to characterize the calcium-dependent interaction between intrinsically generated autonomous spiking and extrinsically generated glutamatergic synaptic transmission in shaping mitochondrial oxidant stress in substantia nigra dopaminergic neurons that are vulnerable in Parkinson's disease.

Body

Key Research Accomplishments:

"Glutamate Signaling and Mitochondrial Dysfunction in Models of Parkinson's Disease"

We have made excellent progress toward our specific aims. This progress is summarized below.

Specific Aim 1: *To characterize interactions between N-methyl-d-aspartate (NMDA) glutamate receptors and Cav1 calcium channels in regulating intracellular calcium and mitochondrial oxidant stress of SNc dopaminergic neurons.*

Progress: Our working hypothesis was that dendritically localized NMDA receptor (NMDAR) opening can significantly elevate cytosolic calcium concentration and induce the release of calcium from endoplasmic reticulum (ER) stores, increasing local and global mitochondrial oxidant stress.

- We have found that antagonizing NMDARs decreases mitochondrial oxidant stress of SNc DA neurons in brain slices. Elevating NMDAR activity by bath application of low micromolar concentrations of NMDA dramatically elevates mitochondrial stress.
- The chronic antagonism of L-type channels in vivo by subcutaneous administration of isradipine (with osmotic minipumps) for 10 days led to a dramatic lowering in mitochondrial oxidant stress in SNc DA neurons. This reduction was accompanied by a reduction in cytosolic calcium oscillations but no change in pacemaking rate, in agreement with acute antagonism of L-type channels.
- We have not found evidence that dendritic NMDAR activation triggers a localized release of calcium from ER stores.
- A major effort has been made to determine the intrinsic calcium buffering capacity of SNc DA neurons. This is critical to the interpretation of all of our calcium imaging experiments and to our approaches examining calcium-induced-calcium-release. We have developed a new strategy that is based upon published protocols and verified that the intrinsic calcium buffering capacity of SNc DA neurons is very low (below 100).
- Our attempts to drive NMDARs using two photon uncaging (2PLU) of glutamate at dendritic sites encountered an experimental problem. Because the location of synapses cannot be readily determined visually in normal brain slices, uncaging glutamate sometimes created large responses and sometimes it did not. We have now developed a mapping strategy with the software controlling our scan head that will allow hot spots (presumed synapses) to be determined quickly. An alternative approach we are developing involves a mesencephalon cell culture model. Although this model is not perfect, it allows the visualization of synaptic sites and allows ready visualization of mitochondria in dendrites; in brain slices, this has proven to be difficult, even with 2PLSM. In these model systems, we are testing additional optical probes that will allow us to monitor mitochondrial calcium uptake during synaptic stimulation.
- The NMDA receptors expressed by SNc dopaminergic neurons are widely thought to have GluN2D subunits. Recently, Dr. Steven Traynelis at Emory University has developed a new class of antagonists that specifically target these subunits and those of the GluN2C variety. We have established a collaboration with Dr. Traynelis to test the impact of these compounds on PPN and STN synaptic responses evoked with optogenetic techniques (see below).
- As described above, we developed an *in vitro* model to study SNc dopaminergic neurons to allow dendritic regions where glutamatergic synapses are formed to be studied more quantitatively. These studies revealed that mitochondrial oxidant stress steadily rose with

distance from the cell body. Furthermore, antagonists of L-type channels eliminated differences with somatic mitochondria, suggesting that as the diameter of the dendrite decreases and mitochondrial density falls oxidant stress rises. This helps to understand why dendrites are exquisitely sensitive to stress. In addition, we found that the formation of intracellular inclusions created by extracellular deposition of alpha-synuclein fibrils further increased mitochondrial oxidant stress. However, this oxidant stress was sensitive to treatment with N-acetyl cysteine, suggesting it was coming from the cytosol. Indeed, expression of a novel cytosolic variant of roGFP revealed that cytosolic oxidant stress was dramatically elevated by inclusion formation. This stress is likely to by lysosomal in origin. This provides an important an important clue about the interaction between Cav1.3 channels, NMDA receptors and alpha synuclein in the evolution of PD. It also suggests that strategies aimed at diminishing alpha synuclein aggregation should work in concert with those aimed at diminishing Ca^{2+} mediated mitochondrial oxidant stress.

Specific Aim 2: ***To characterize interactions between metabotropic glutamate receptors and Cav1 calcium channels in regulating intracellular calcium and mitochondrial oxidant stress of SNc dopaminergic neurons.***

Progress: Our working hypothesis is that dendritic metabotropic glutamate receptor type 5 (mGluR5) signaling induces ER calcium release that increases mitochondrial oxidant stress both locally and globally. Furthermore, we hypothesize that diminishing the calcium content of the ER stores by antagonizing Cav1 calcium channels will reduce the mitochondrial stress created by mGluR5 activation.

- Contrary to our hypothesis, antagonizing mGluR1/5 receptors had no effect on basal mitochondrial oxidant stress in SNc DA neurons in brain slices. Moreover, it had no effect on basal pacemaking or intracellular calcium oscillations.
- However, blocking IP3 receptors or ryanodine receptors significantly diminished mitochondrial oxidant stress. Moreover, intracellular calcium oscillations appear to be attenuated following ER store depletion. These results suggest that ER stores and calcium-induced calcium release (CICR) are important factors in determining intracellular calcium oscillations and mitochondrial stress but that in the brain slice mGluRs are not active enough to modulate this mechanism. To test this hypothesis, we will apply exogenous mGluR1/5 agonists while monitoring intracellular calcium oscillations and pacemaking. In addition, we will determine the effect of mGluR5 agonists on mitochondrial oxidation.
- A key part of our current model is that calcium entry through Cav1.3 channels triggers CICR. This CICR process should be amplified by mGluR1/5 stimulation. We also hypothesize that CICR is critical to the influx of calcium into mitochondria - a necessary condition for the elevation of oxidant stress. We have verified this using a blocker of the mitochondrial uniporter (RU360). To better nail this down, we are developing optical probes that will allow us to monitor calcium entry into the mitochondrial matrix as well as calcium exodus from the ER. We are making excellent progress toward this goal and have functional ER Ca^{2+} indicators packaged in an AAV vector suitable for *in vivo* injection.

Specific Aim 3: ***To characterize responses of SNc dopaminergic neurons to normal and pathological patterns of activity in glutamatergic fibers arising from the pedunculopontine nucleus (PPN).***

Progress: Anatomical studies have shown that the principal glutamatergic input to SNc dopaminergic neurons arises from neurons in the PPN. Our working hypothesis is that these inputs will produce responses in SNc dopaminergic neurons similar to those produced by exogenous glutamate application.

- Our initial strategy was to use optogenetic approaches to study the PPN input to SNc DA neurons. We discovered that because channelrhodopsin2 (ChR2) rapidly desensitizes, burst stimulation of axons was giving us spurious results. As a consequence, we have taken two new approaches.
- First, we developed a brain slice preparation that preserves the connectivity between the PPN and the SNc, allowing us to electrically stimulate PPN axons and measure synaptic responses in SNc DA neurons. These studies have revealed that 1) the PPN glutamatergic synapse is a high release probability, depressing synapse; 2) there is a robust NMDAR component to the PPN evoked synaptic response; 3) antagonism of ionotropic glutamate receptors essentially eliminates the response to PPN stimulation, suggesting that nicotinic receptors are not prominent mediators of the response to activation of cholinergic neurons in the PPN.
- Second, we have developed optogenetic tools necessary to selectively activate the cholinergic and glutamatergic inputs to SNc DA neurons using cell-type specific Cre transgenic mice. Although we cannot stimulate these systems at high frequency, we can use this approach to map these inputs onto the dendritic tree of SNc DA neurons and characterize how these inputs are modulated. We can also use this approach to activate these inputs selectively and monitor alterations in pacemaking, calcium oscillations and mitochondrial stress. These experiments have yielded surprising results. First, the PPN glutamatergic input to SNc neurons was localized to the proximal 100 microns of the dendritic tree, whereas the subthalamic nucleus (STN) input appears to be more distal. The impact of each of these synapses on intracellular Ca^{2+} and local mitochondrial oxidant stress is now being investigated. Second, the cholinergic input from the PPN evoked smaller, nicotinic acetylcholine receptor (nAChR) synaptic responses. To determine the impact of nicotine on the nAChR responses we bath applied it at physiologically meaningful concentrations (100-500 nM). This dose had no effect on basal pacemaking, but did evoke a small (30-50 pA) inward current. Astonishingly, this inward current was accompanied by a dramatic reduction in the amplitude of intracellular Ca^{2+} oscillations attributable to Cav1.3 L-type channels. This effect was not mediated by a direct channel block. As expected from this modulation, mitochondrial oxidant stress was significantly lowered by nicotine. To our knowledge, this provides the first parsimonious explanation for the long known reduction in PD risk that accompanies tobacco smoking. We are currently pursuing the receptor subtype mediating this effect and its mechanism. It is our working hypothesis that the receptor is an alpha6beta2 containing receptor and its effect is mediated by activation of calcineurin. If the former is true, it would point to a therapeutic strategy that lacked abuse potential (alpha4beta2-mediated) or peripheral toxicity (alpha3-mediated).

Specific Aim 4: ***To characterize mitochondrial function and glutamatergic signaling of SNc dopaminergic neurons in a model of early stage PD.***

Progress: Our working hypothesis is that in the early stages of PD, pathological bursting emerges in glutamatergic neurons projecting to the SNc, leading to an elevation in extracellular glutamate and mitochondrial oxidant stress in the remaining dopaminergic neurons.

- The in vitro experiments with partially lesioned animals (using intrastriatal 6-OHDA injections) have proven more difficult than we anticipated. SNc DA neurons in these animals either looked normal in redox status or looked severely compromised. There was no evidence in the healthy neurons of elevated extracellular glutamate. The percentage of cells that looked normal was much lower than the percentage of nominally normal neurons judged by tyrosine hydroxylase (TH) immuncytochemistry. Our interpretation of these studies is that many of the neurons in the SNc of the lesioned animals are very stressed and vulnerable to the added stress of brain slicing, but have normal TH levels. To test this hypothesis, we will give isradipine after establishment of the lesion; animals will be treated for a minimum of one week prior to slicing. Our experience is that this treatment dramatically improves the viability of SNc DA neurons (see above).

- To complement these studies, acute treatment with isradipine and/or MK-801 (an NMDAR antagonist) will be performed.
- Collaborative studies with Dr. Greengard are underway but have not yielded definitive results as of yet.

Specific Aim 5: ***To characterize interactions between glutamate receptors and Cav1 calcium channels in influencing mitochondrial oxidant stress in a genetic model of PD.***

Progress: These experiments have not been initiated yet.

Reportable Outcomes:

Publications:

Dryanovski DI, Guzman JN, Xie Z, Galteri DJ, Volpicelli-Daley LA, Lee VMY, Miller RJ, Schumacker PT, and Surmeier DJ (2013) Calcium entry and α-synuclein inclusions elevate dendritic mitochondrial oxidant stress in dopaminergic neurons. Journal of Neurosciene (in revision).

Surmeier DJ, Guzman JN, Sanchez-Padilla J, Schumacker PT (2011) The role of calcium and mitochondrial oxidant stress in the loss of substantia nigra pars compacta dopaminergic neurons in Parkinson's disease. Neuroscience 198:221-231.

Surmeier DJ, Guzman JN, Sanchez-Padilla J, Goldberg JA (2011) The origins of oxidant stress in Parkinson's disease and therapeutic strategies. Antioxidants & redox signaling 14:1289-1301.

Ilijic E, Guzman JN, Surmeier DJ (2011) The L-type channel antagonist isradipine is neuroprotective in a mouse model of Parkinson's disease. Neurobiology of disease 43:364-371.

Surmeier DJ, Guzman JN, Sanchez-Padilla J, Goldberg JA (2010) What causes the death of dopaminergic neurons in Parkinson's disease? Prog Brain Res 183:59-77.

Conclusion:

The Conclusion is summarized in the Progress sections for each Aim listed above.